mRNA electroporation of dendritic cells with WT1, survivin and TriMix (a mixture of caTLR4, CD40L, and CD70)

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Abstract

The immune system is a crucial player in the development of cancer. Once it is in imbalance and immunosuppressive mechanisms supporting tumor growth take over control, dendritic cell immunotherapy might offer a solution to restore the balance. There are several methods to manufacture dendritic cells but none of them has yet proven to be superior to others. In this chapter, we discuss the methodology using electroporation of mRNA encoding Wilms' tumor gene 1, survivin and TriMix (mixture of caTLR4, CD40L, and CD70) to simultaneously load and mature dendritic cells.

Key words

mRNA, electroporation, dendritic cell, WT1, survivin, TriMix

Running head

Dendritic cell electroporation

1. Introduction

It becomes increasingly clear that the immune system is important in the development of cancer. Once initial neoplastic cells arise, the host immune system strives to control their progression mainly through the presence of effector T cells (Teff) and NK cells. This stage is called the cancer immune surveillance. One of the different innate immune cells controlling tumor development during the immune surveillance stage is the dendritic cell (DC). These are professional antigen presenting cells, that can capture tumor-associated antigens (TAA), expressed by cancer cells, in their immature state (DCi) in a tumor milieu. Upon ingestion of the TAA and in the presence of an appropriate "danger" signal, for example inflammatory cytokines, DC start to mature (DCm) and migrate to the lymph nodes, where they make contact with and activate T cells that will then traffic to the target organ (the tumor) to selectively destroy the neoplastic cells. If this process is successful, the neoplastic cells are either eliminated or come to reside in a dormant state (elimination *versus* equilibrium). However, the problem is that tumor cells might also be capable to escape from the control of the immune system, start proliferating and cause the tumor to become clinically apparent ***(1)***. Tumor immune escape is facilitated by two main pathways: 1/ mechanisms that eventually cause antigen loss and 2/ the infiltration of immunosuppressive cells, which are attracted to the tumor microenvironment due to the sustained secretion of chemokines and cytokines (TGF-β (tumor growth factor beta), IL-10 (interleukin-10), VEGF (vascular endothelial growth factor), galectin-1,…) produced by the tumor and immune cells present in and around the tumor. These immunosuppressive cells will in turn also start producing several mediators that will strengthen their effects and will cause more negative feedback on other cells, leading to a downward spiral of effects all contributing to the escape of tumor cells from the immune surveillance. There are several players within this group of immunosuppressive cells. However the most currently relevant cells in pelvic gynecological tumors are the regulatory T

cells (Treg), the myeloid-derived suppressor cells (MDSC) and the tumor-associated macrophages (TAM) ***(2, 3)***.

DC immunotherapy is an attempt to increase the number of potent DCm (and consequently tumor-specific T cells) in order to shift the balance from immunosuppression towards cancer immune surveillance ***(4)***. At this level, there is room for improvement. In a recent review, we summarized in a non-exhaustive list possible strategies that can be used to improve the effectiveness of DC ***(5)***. At present, no method has proven to be superior to another. Therefore, we opted for a combination of three different routes of improvement: 1/ the use of two predefined TAA, Wilms' tumor gene 1 (WT1) and survivin, both linked to a DC-LAMP targeting signal to obtain both $CD4^+$ and $CD8^+$ T cell induction; 2/ the use of TAA at the mRNA level for DC electroporation; 3/ the use of TriMix mRNA to simultaneously mature DCi.

The choice of two TAA is explained by the rational that the risk of immune escape is reduced and the group of tumors that can be targeted is broadened ***(6)***. The *in vitro* work of several research groups has suggested that RNA transfection of TAA is an effective method to generate immunostimulatory DCs, regardless of the patients HLA-type ***(4,7)***. Further modifications of the coding sequence by adding lysosomal targeting sequences (e.g., DC-LAMP) lead to the presentation of antigenic peptides in the context of both HLA class I and class II ***(8-10)***. Our research group has proven the safety and feasibility of DC immunotherapy in uterine and ovarian tumors (*WT1* mRNA-loaded DCs) ***(11,12)***. In a recent review by our research group and the group of Kris Thielemans, the use of TriMix was explained. In brief, TriMix is constituted of three different mRNAs: caTLR4 [constitutively active Toll-like receptor 4], CD40L, and CD70. Transfection with TriMix leads to mature, cytokine/chemokine-secreting DC that are capable of stimulating naive T cells and support their proliferation. The technique is fast and results in semi-mature DC 3.5 hours after

electroporation. As a result, TriMix DC will mature and secrete most of their immunostimulatory cytokines and chemokines *in vivo* after human injection ***(6,13)***.

This chapter will especially outline the procedure of the electroporation. Details will be given on how to handle electroporation cuvettes and mRNA, what steps should be followed consecutively to obtain an optimal result and what should be avoided. The protocol will also give you an insight in how to work with large volume culture material (ULA).

2. Materials

2.1. Reagents

1. Supplemented RPMI: RPMI, 1% human autologous plasma (heat-inactivated 30 min at 56°C), IL-4 (500 IU/mL), GM-CSF (1000 IU/mL)
2. DPBS + EDTA: Dulbecco's phosphate-buffered saline with Ethylenediaminetetraacetic acid (460 mg/L)
3. OptiMEM, Reduced Serum Medium, no phenol red
4. Rapamycin
5. *WT1.DC-LAMP* mRNA
6. *survivin.DC-LAMP* mRNA
7. *TriMix* mRNA

2.2. Equipment

1. Warm water bath (56°C)

2. Tabletop centrifuge consisting of a rotor and buckets capable of holding 50 mL tubes and reaching at least 300 g
3. 5% CO_2 humidified incubator
4. 5 and 50 mL conical tubes
5. 2 ml cryovials
6. 4 mm electroporation cuvettes
7. RNase free tips of 40 to 200 µL and of 0.5 to 10 µL (*see* Note 1)
8. Corning® CellSTACK® cell culture chambers
9. Electroporation device
10. Bürcker chamber (microscope counting)

3. Methods

3.1. mRNA electroporation of immature dendritic cells

The procedure is for 400 x 10^6 to 600 x 10^6 DCi in total (*see* Note 2). Step 1-6 are carried out in advance of step 7 (*see* Note 3):

1. Fill the ULA with 200 mL supplemented RPMI (*see* Note 4 and 5)
2. Fill a 50 ml conical tube with supplemented RPMI
3. Centrifuge the *WT1.DC-LAMP*-mRNA, *survivin.DC-LAMP*-mRNA and *TriMix*-mRNA at maximal speed during 5 seconds
4. Take out 60 µg *WT1.DC-LAMP*-mRNA + 60 µg *TriMix*-mRNA and transfer into cryovial A; take out 60 µg *survivin.DC-LAMP*-mRNA + 60 µg *TriMix*-mRNA and transfer into cryovial B

5. Complete cryovial A and B with OptiMEM until a final volume of 200 µL has been reached
6. The process described in step 4 and 5 is sufficient to electroporate 50 x 10^6 DCi. Repeat the procedure for cryovial A for half of the total amount of DCi. Repeat the procedure for cryovial B for half of the total amount of DCi
7. Dissolve 50 x 10^6 immature DC (DCi) in 10 ml OptiMEM in a 50 ml conical tube
8. Repeat this step until all DCi are used
9. Centrifuge DCi at 300 g during 10 minutes at room temperature
10. Discard the supernatant
11. Dissolve each DCi pellet in 400 µl OptiMEM in the 50mL conical tube
12. Transfer the content of one 50 mL conical tube into one electroporation cuvette
13. Rinse each 50 mL conical tube with one mRNA mix (200 µL volume) to collect all remaining DCi (step 5)
14. Transfer the 200 µL into the electroporation cuvette. Final volume in electroporation cuvette is now 600 µL (*see* Note 6)
15. Mix contents very gently by repeated pipetting (*see* Note 7)
16. Electroporate at 300 V and 450 µF, using exponential decay
17. Transfer the contents of the electroporation cuvette into the ULA (*see* Note 8)
18. Rinse the cuvette twice with 800 µL RPMI medium from the 50mL conical tube prepared in step 2.
19. Repeat step 7 until 13 for all 50 mL conical tubes

20. Gently shake the ULA to homogenate the cells
21. Add 100 nM rapamycin to the ULA
22. Place the ULA at 37°C with 5% CO_2 during 3,5 hours

3.2. Preparation of mature dendritic cells

1. Gently shake the ULA
2. Empty the ULA with a 25 mL pipet into 50 mL conical tubes
3. Add 75 mL DPBS + EDTA to the ULA (*see* Note 9)
4. Place the ULA at 37°C with 5% CO_2 during 15 minutes
5. Gently shake the ULA
6. Empty the ULA with a 25 mL pipet into 50 mL conical tubes
7. Rinse the ULA twice with 75 mL supplemented RPMI
8. Empty the ULA with a 25 mL pipet into 50 mL conical tubes
9. Centrifuge all 50 mL conical tubes at 300 g during 10 minutes at room temperature
10. Discard the supernatant
11. Pool the content of all 50 mL conical tubes in a total volume of 40 mL supplemented RPMI
12. Count the amount of mature DC (DCm) in the Bürcker chamber
13. Centrifuge DCm at 300 g during 10 minutes at room temperature
14. Discard the supernatant
15. DCm can now be used in further experiments (*see* note 10)

4. Notes

1. RNA is fragile and subject to the activity of nucleases, particularly RNases. Therefore, use RNase-free material (indicated on the packing of disposable material) when handling pure mRNA (before electroporation) and work with sterile technique. Always use gloves and never touch anything with bare hands, since they contain nucleases. Once the mRNA is electroporated into the DCi, non-RNase free material can be used.
2. DCi can be obtained from culturing monocytes into DC. In our hands, peripheral blood mononuclear cells are cultured in cell factories (plastic adherence) over 6 days. IL-4, GM-CSF and autologous plasma are added to the cell factories on day 0, 2 and 4. At day 6, the cells have been differentiated into DCi. Several other protocols have been described to generate DCi ***(5)***.
3. Before you start the procedure, make sure that your filter tips can reach the bottom of the electroporation cuvette
4. RPMI should be at room temperature before use
5. Supplemented RPMI can be kept at 4°C after filling the ULA
6. When filling the electroporation cuvette, try not to overfill. The fluid level cannot overreach the electrode plates
7. When working in the electroporation cuvettes, try not the create air bubbles because they will prevent effective electroporation
8. After electroporation, cells can clot. Make sure you do not use a filter tip with a small diameter to empty the electroporation cuvette - tips from 0.5 to 10 µL are too small. Preferably use a filter tip from 200 to 1000 µL and use it at 800 µL. After rinsing, the electroporation cuvette, you can use a small pipette filter tip of 0.5 to 10 µL to empty the cuvette

9. Never use DPBS + EDTA longer than 15 minutes, because it will become toxic for the DC
10. All work should be performed in a biological safety cabinet in order to work sterile.

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